Plant Cell, Tissue and Organ Culture **77:** 157–163, 2004.

Influence of media components and pH on somatic embryo induction in three genotypes of soybean

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Received 6 June 2003; accepted in revised form 22 August 2003

Key words: embryogenesis, *Glycine max*, immature cotyledons

Abstract

The influence of media components on the initiation of somatic embryogenesis in three genotypes of soybean was investigated. The following genotypes were used: Iroquois, Macon, and Savoy. Media modifications included sucrose concentration, type and concentration of auxin at two pH levels, and pH level independently. Immature cotyledons were used as the source of explant. Cotyledons were placed on a medium containing MS salts, B5 vitamins, sucrose, and auxin. Gelrite (0.2%) was used as the solidifying agent. Sucrose concentrations of 1, 2, 3, 4.5, or 6% were used. The auxins used included 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA) with each at concentrations of 45.2, 90.4, 135.7, 180.9, and 226.2 μM. The pH of each the media was adjusted to either 5.7 or 7.0 with 1 N NaOH. In an additional experiment, the effect of the two pH levels, 5.7 and 7.0, was investigated independently. Overall, the frequency of somatic embryogenesis significantly varied among the different genotypes used in this study, with Iroquois showing the highest response. Frequency of somatic embryogenesis also varied in response to the different treatments used, including sucrose and auxin. The highest initiation (91.7%) and mean number of somatic embryos per responding explant (14.9) of Iroquois was observed in a medium containing 2% sucrose. The highest initiation (97.1%) and mean number of somatic embryos per responding explant (19.5) was observed in Iroquois on 135.7 μM 2,4-D and Savoy on 135.7 μM 2,4-D, respectively, for the auxin by pH level experiment. No significant differences were observed among the two pH treatments used.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid; NAA – naphthaleneacetic acid

Introduction

Soybean [*Glycine max* (L.) Merrill] has been regenerated *in vitro* via both shoot organogenesis and somatic embryogenesis. Regeneration via shoot organogenesis has been reported using several explant sources such as the cotyledonary or primary leaf nodes (Barwale et al., 1986; Kim et al., 1990) and primary leaves of seedlings (Wright et al., 1987). Regeneration via somatic embryogenesis has been reported using immature cotyledons (Lippmann and Lippmann, 1984; Lazzeri et al., 1985; Ranch et al., 1985; Parrott et al., 1989). *In vitro* culture systems can be used along with various methods of genetic manipulation and/or improvement which can utilize either method of regeneration. One of the most important systems that facilitate genetic engineering and *in vitro* propagation of soybean is the somatic embryo culture system (Samoylov, 1998). Embryogenic suspension cultures of soybean have been successfully used for particle bombardment (Finer and McMullen, 1991; Parrott et al., 1994; Stewart et al., 1996; Santarem and Finer, 1999). Yet, these successes have been limited to those genotypes that are amenable to embryogenic culture induction in tissue culture.

Proliferative embryogenic cultures provide suitable and convenient target tissues for genetic transformation, although initiation and maintenance of these cultures are time-consuming and labor-intensive (Santarem et al., 1997). Induced somatic embryos develop directly on the explant in a matter of a few weeks, and then can be targeted for transformation using either *Agrobacterium* or the particle gun (Santarem et al., 1997). Unfortunately, initiation of somatic embryos in soybeans is inefficient (Santarem et al., 1997; Simmonds and Donaldson, 2000; Meurer et al., 2001). Recently, Tomlin et al. (2002) have reported that somatic embryogenesis is positively associated with lower maturity groups of soybean. Therefore, efforts to study factors which influence somatic embryo initiation are useful to enhance the efficiency of initiation of somatic embryogenesis.

Various studies have reported on the influence of factors such as auxin (Lazzeri et al., 1987, 1988; Komatsuda and Ohyama, 1988), sucrose (Lazzeri et al., 1987, 1988; Komatsuda et al., 1991), pH (Lazzeri et al., 1987; Santarem et al., 1997), and genotype (Komatsuda and Ohyama, 1988; Parrott et al., 1989; Komatsuda et al., 1991; Tomlin et al., 2002) on somatic embryogenesis in soybeans. As new genotypes are released from breeding programs, they are also being targeted for genetic manipulation/engineering efforts. Thus, developing an *in vitro* regeneration system is a prerequisite for such manipulations. In the present study, the influence of media variables, including sucrose concentration, pH level, and auxin type and concentration on initiation of somatic embryos in three new genotypes of soybean has been investigated.

Materials and methods

Plant material

Seedlings of the following three soybean genotypes, Iroquois, Macon, and Savoy, were grown in the greenhouse at 26 ± 1 °C. Immature pods containing cotyledons of 3–6 mm in length were collected, surface-sterilized in 1.1% sodium hypochlorite solution (20% clorox® commercial bleach) containing 3–4 drops of Tween-20 for 25 min, and rinsed three times with sterilized-deionized water. Immature cotyledons were excised, and embryonic axes were removed according to Lazzeri et al. (1985).

Culture conditions and basal medium

Cotyledons were placed with the adaxial surface oriented upward on approximately 35 ml of solidified medium in 15 × 120-mm petri dishes. Cultures were incubated under a 23-h photoperiod provided by 40 W cool-white fluorescent tubes illuminating 40–60 μmol m^{-2} s^{-1} light intensity at 25 ± 1 °C for 4 weeks in a controlled environment. The basal culture medium consisted of MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 3% sucrose, 180.9 μM 2,4-D, and solidified with 0.2% gelrite. Prior to autoclaving, the pH level was adjusted to 7.0 with 1 N NaOH.

Influence of various medium components and pH on somatic embryogenesis

To determine the influence of sucrose concentration, explants were incubated on the basal medium, except for sucrose whereby the following five concentrations were used instead, including 1.0, 2.0, 3.0, 4.5, and 6.0%.

To investigate the effect of the pH of the medium, explants were incubated on the basal medium, but the pH level was adjusted to either 5.7 or 7.0 with 1.0 N NaOH.

To determine the influence of auxin type and concentration, explants were incubated on the basal medium, but containing either 2,4-D or NAA, each supplemented at five concentrations (45.2, 90.4, 135.7, 180.9, and 226.2 μM). The pH was adjusted to either 5.7 or 7.0 with 1 N NaOH.

Experimental design and data analysis

All experiments were set-up in a randomized complete block design with the date of culture representing a single block. A total of six culture dates were used. For all treatments, one petri dish per treatment per genotype per day was used with each petri dish containing eight to 12 cotyledon-halves. At least five plates per treatment per genotype were used.

Plates were scored for number of explants initiating somatic embryos and for number of somatic embryos per responding explant after 4 weeks of culture. Data were analyzed using SAS General Linear Model (GLM) and means were compared using least significant difference (LSD) procedures (SAS Institute Inc., Cary, NC).

Table 1. Influence of sucrose concentration on percent initiation of somatic embryos and number of somatic embryos per responding explant

Sucrose concentration (%)	% Immature cotyledons initiating somatic embryos (mean ± SD)			No. somatic embryos/responding explant (mean ± SD)		
	Genotype			Genotype		
	Iroquois	Macon	Savoy	Iroquois	Macon	Savoy
1.0	81.9 ± 6.3	47.2 ± 8.7	15.0 ± 22.4	8.2 ± 2.8	3.9 ± 3.1	1.4 ± 2.2
2.0	91.7 ± 7.5	63.9 ± 27.7	54.2 ± 22.3	15.0 ± 7.8	6.5 ±1.8	5.3 ± 2.5
3.0	86.0 ± 22.4	77.1 ± 15.8	47.2 ± 27.7	13.3 ± 5.4	11.3 ± 4.5	6.2 ± 5.0
4.5	75.0 ± 22.4	55.0 ± 26.8	36.1 ± 12.6	10.4 ± 4.7	6.4 ±1.8	10.3 ± 6.2
6.0	50.0 ± 19.0	40.4 ± 22.7	27.8 ± 13.6	5.7 ± 2.9	3.4 ±2.0	7.9 ± 3.2

For each sucrose concentration treatment, one petri dish per treatment per genotype was used with each petri dish containing eight to twelve cotyledon-halves. At least five plates per treatment per genotype were used.

Results and discussion

Influence of sucrose concentration on initiation of somatic embryogenesis

Somatic embryos were initiated in all three genotypes at all five sucrose concentrations with the lowest initiation frequency (15%) observed at 1% sucrose for the genotype Savoy (Table 1). Overall, percent somatic embryo initiation for all three genotypes was above 50% depending on the sucrose concentration used, with both Iroquois and Macon responding above 75% at 2 and 3% sucrose, respectively (Table 1). Barwale et al. (1986) reported the highest frequency of embryogenesis (65%) with genotype Williams 82. In a study conducted by Parrot et al. (1989), the highest percent of cotyledons forming somatic embryos was 76% in genotype Century. In a later study by Parrott et al. (1993), initiation frequencies among genotypes tested ranged from 46% for PI 417138 to 94% for Centennial. In the present study, over all concentrations of sucrose and all three genotypes, percent initiation of somatic embryogenesis ranged from 15 to 92% (Table 1).

Cultures initiated on lower sucrose concentrations (1 and 2%) tended to produce higher amounts of friable callus than elevated concentrations of sucrose (4.5 and 6%). Highly significant ($p < 0.0001$) effects for percent initiation of somatic embryogenesis were observed for both genotype and sucrose concentration. There was no genotype × sucrose interaction for percent initiation of embryogenesis. Previous studies reported similar findings with genotype, sucrose concentration, and genotype × sucrose concentration all highly significant (Komatsuda et al., 1991). In the present study, all three genotypes exhibited the highest percent initiation at 2 and 3% sucrose (Table 1). Both Lippmann and Lippmann (1984) and Lazzeri et al. (1988) reported similar results to those obtained in this study with Iroquois and Macon, whereby lower sucrose concentrations produced the highest percent initiation of embryogenesis. Lazzeri et al. (1987) reported an increase in the efficiency of embryogenesis as the sugar concentration decreased from 12 to 1.5%, with 3% sucrose producing the highest frequency in genotype J103. Lippmann and Lippmann (1984) did not observe embryogenesis with sucrose concentrations above 2% in genotype Maple Arrow. The highest percent initiation was observed at 2% sucrose in Iroquois (Table 1). This treatment resulted in 92% initiation.

Highly significant effects for mean number of somatic embryos per responding explant were observed for genotype ($p < 0.0048$), sucrose concentration ($p < 0.0001$), and genotype × sucrose interaction ($p < 0.002$). Overall concentrations of sucrose and all genotypes, mean number of somatic embryos per responding explant ranged from 1.43 to 14.95 (Table 1). Iroquois and Macon had the highest number of somatic embryos per responding explant at 2 and 3% sucrose; whereas the highest response for Savoy was observed at 4.5 and 6% sucrose (Table 1). The mean number of somatic embryos per responding explant for both Iroquois and Macon increased as the sucrose concentration in the medium increased from 1 to 3% sucrose, but then decreased as sucrose concentration increase further (4.5–6.0%). Lazzeri et al. (1987) also reported a decrease in mean number of somatic

embryos per responding cotyledon as the sucrose concentration increased from 1.5 to 12% for soybean genotype J103. Komatsuda et al. (1991) obtained high numbers of somatic embryos on two low sucrose concentrations (0.5 and 1%) for 16 soybean genotypes. This was in agreement to the results obtained in this study for Iroquois and Macon, whereby the lower concentrations (2 and 3%) of sucrose used produced higher numbers of somatic embryos (Table 1). In the present study, the lowest sucrose concentration (1%) responded similarly to the higher concentrations (4.5 and 6.0%) (Table 1). The highest mean number of somatic embryos per responding explant (14.95) was observed at 2% sucrose in Iroquois (Table 1).

Influence of medium pH level on initiation of somatic embryogenesis

Somatic embryos were induced in all three genotypes at both pH 5.7 and 7.0. Significant effects for percent initiation of embryogenesis was observed for genotypes, with Iroquois showing the highest percent initiation (80%) at pH 7.0 (Table 2). The effect of pH was not significant for percent initiation of somatic embryogenesis. Moreover, significant effects were observed for mean number of somatic embryos per responding explant. Studies involving somatic embryogenesis of soybean have reported the use of a pH range of 5.7–5.9 (Lazzeri et al., 1987; Hartweck et al., 1988; Shoemaker et al., 1991) and pH 7.0 (Bailey et al., 1993; Li and Grabau, 1996). Previous studies observed differences among pH levels with pH 7.0 producing the highest frequency of initiation with genotypes Jack, Thorne, and Resnik (Santarem et al., 1997). Santarem et al. (1997) reported an average of 44.2 embryos per explant in cv. Jack, while the other genotypes produced a maximum of 13 embryos per explant. In the present study, the highest production was 9.6 embryos per explant at pH 5.7 in genotype Iroquois (Table 2). Lazzeri et al. (1987) reported no differences in frequency of embryogenesis for pH levels ranging between 5.0 and 7.0. This was similar to results obtained in this study.

It has been suggested that the effect of pH on somatic embryo initiation may be related to auxin uptake in cultured explants, and that a pH of 7.0 may facilitate slower and more gradual uptake of 2,4-D at relatively high levels (Santarem et al., 1997). Edwards and Goldsmith (1980) have reported increased auxin uptake in maize coleoptiles with decreasing pH. Davies and Rubery (1978) also have found that auxin uptake increased as the external pH is lowered in stem segments of *Pisum sativum* L. Perhaps, exogenous 2,4-D levels in the soybean culture medium used in this study are very high, and as a result by lowering the pH level from 7.0 to 5.7, there is no significant influence on auxin uptake.

Influence of auxin type and concentration at two pH levels

Somatic embryos were initiated in all three genotypes in the presence of both auxins (2,4-D and NAA) at all concentrations and pH levels used. However, varying responses were observed among the different treatments. Somatic embryos were not induced on media lacking either 2,4-D or NAA. Significant effects for concentration, auxin × concentration, genotype × auxin, and genotype × concentration two-way interactions, and genotype × auxin × concentration three-way interaction were observed for percent initiation (data not shown). Shoemaker et al. (1991) reported embryogenic response reached optimum levels when 2,4-D concentrations ranged from 38 to 132 μM. In the present study, the highest percent initiation across the three genotypes was observed with 90.4 μM 2,4-D which was the mean for the range of 2,4-D concentrations reported by Shoemaker et al. (1991). NAA produced a slightly higher average percent initiation (84.8%) than 2,4-D (82.2%), whereas other studies observed the highest percent initiation with 2,4-D (Lippmann and Lippmann, 1984; Barwale et al., 1986). For genotype × auxin × concentration effects, the highest percent initiation (97%) was observed in Iroquois on a medium containing 135.7 μM 2,4-D (Table 3). This is in agreement with previous studies where it has been reported that 2,4-D was highly potent for somatic embryogenesis in soybean (Lazzeri et al., 1987).

Significant effects for auxin, concentration, auxin × concentration, and genotype × auxin two-way interactions were observed for mean number of somatic embryos per responding explant (data not shown). In addition, a significant three-way interaction for genotype × auxin × concentration was also observed for mean number of somatic embryos per responding explant. The mean number of somatic embryos per responding explant showed the highest response, 19.5 somatic embryos per explant, at 135.7 μM 2,4-D in Savoy (Table 3). Shoemaker et al. (1991) reported mean number of somatic embryos per explant reached optimum levels when 2,4-D concentrations

Table 2. Influence of pH level on initiation of somatic embryos and number of somatic embryos per responding explant

pH	% Immature cotyledons initiating somatic embryos (mean ± SD)			No. somatic embryos/responding explant (mean ± SD)		
	Genotype			Genotype		
	Iroquois	Macon	Savoy	Iroquois	Macon	Savoy
5.7	79.2 ± 13.7	54.7 ± 22.0	56.7 ± 17.3	9.6 ± 3.2	7.2 ± 2.3	6.5 ± 3.5
7.0	80.3 ± 12.3	57.2 ± 19.8	44.4 ± 27.2	8.0 ± 2.3	6.2 ± 2.3	7.6 ± 5.3

For each pH treatment, one petri dish per treatment per genotype was used with each petri dish containing eight to twelve cotyledon-halves. At least five plates per treatment per genotype were used.

Table 3. Effect of auxin type and concentration on initation of somatic embryos and number of somatic embryos per responding explant

Auxin	Conc. (μM)	% Immature cotyledons initiating somatic embryos (mean ± SD)			No. somatic embryos/responding explant (mean ± SD)		
		Genotype			Genotype		
		Iroquois	Macon	Savoy	Iroquois	Macon	Savoy
2,4-D	45.2	88.3 ± 10.5	75.5 ± 21.3	80.8 ± 16.2	9.2 ± 4.1	5.0 ± 1.0	7.5 ± 3.4
	90.4	94.0 ± 12.4	92.4 ± 12.4	84.8 ± 15.2	13.8 ± 5.7	10.1 ± 5.7	14.7 ± 7.5
	135.7	97.1 ± 5.3	85.8 ± 13.9	75.0 ± 24.7	16.4 ± 5.2	14.4 ± 5.0	19.5 ± 9.4
	180.9	90.0 ± 6.9	79.5 ± 19.2	75.5 ± 24.6	17.2 ± 5.7	15.9 ± 6.0	14.4 ± 8.7
	226.2	89.0 ± 9.7	81.3 ± 11.8	43.3 ± 24.3	14.3 ± 5.1	14.3 ± 8.3	6.7 ± 3.4
NAA	45.2	64.6 ± 22.9	72.3 ± 25.6	70.0 ± 22.1	2.9 ± 1.2	2.3 ± 0.7	2.7 ± 0.9
	90.4	82.9 ± 9.9	84.1 ± 13.9	85.5 ± 13.7	4.3 ± 1.3	2.9 ± 1.1	4.0 ± 1.3
	135.7	90.2 ± 9.1	90.6 ± 8.4	91.7 ± 11.2	4.6 ± 1.6	4.1 ± 1.5	4.3 ± 1.1
	180.9	85.4 ± 8.8	94.4 ± 7.5	87.5 ± 7.5	5.3 ± 2.1	4.0 ± 0.9	4.6 ± 0.8
	226.2	91.3 ± 9.5	87.2 ± 12.9	92.5 ± 6.2	5.6 ± 2.3	4.3 ± 1.5	4.8 ± 1.3

For each auxin type and concentration treatment, one petri dish per treatment per genotype was used with each petri dish containing eight to twelve cotyledon-halves. At least five plates per treatment per genotype were used.

For percent initiation of somatic embryos, significant differences were observed for date of culture initiation ($p < 0.001$; df= 5; $F = 5.53$) and auxin concentration ($p < 0.0001$; df=4; $F = 10.08$). Significant two-way interactions were observed for date of culture initiation × genotype ($p < 0.0001$; df=10; $F = 3.76$), auxin type × auxin concentration ($p < 0.0001$; df = 4; $F = 13.32$), genotype × auxin type ($p < 0.0001$; df= 2; $F = 18.24$), and genotype × auxin concentration ($p < 0.01$; df= 8; $F = 2.35$). Moreover, a significant genotype × auxin × auxin concentration ($p < 0.001$; df= 8; $F = 3.25$) was also observed.

For number of somatic embryos per explant, significant differences were observed for date of culture initiation ($p < 0.0001$; df= 5; $F = 9.06$), auxin type ($p < 0.0001$; df =1; $F = 388.52$), and auxin concentration ($p < 0.0001$; df =4; $F = 19.63$). Significant two-way interaction for genotype × date of culture initiation ($p < 0.01$; df=10; $F = 2.51$) and genotype × auxin concentration ($p < 0.01$; df = 8; $F = 2.73$) were observed. Moreover, a significant three-way interaction for genotype × auxin × auxin concentration ($p < 0.01$; df =8; $F = 2.42$) was also observed.

ranged from 58 to 132 μM across all genotypes used. In the present study, when NAA was used for somatic embryo initiation instead of 2,4-D, the highest mean number of somatic embryos per responding explant was 5.63 at a concentration of 226.2 μM (42.1 mg l^{-1}) in Iroquois. Lazzeri et al. (1988) reported that the highest number of somatic embryos (4.48) was observed with 12.5 mg l^{-1} NAA. Overall, the mean number of embryos produced on 2,4-D (12.94) was significantly higher ($p < 0.0001$, Table 3) than that produced on NAA (4.03).

Again in this experiment, no significant differences between the two pH levels for percent somatic embryogenesis are observed. This concurs with results obtained in the above experiment. Previous reports indicate that pH levels influence uptake of auxin (Davies

and Rubery, 1978; Edwards and Goldsmith, 1980; Santarem et al., 1997). In this study, the lack of pH influence on frequency of embryogenesis may be due to the genotypes used or to changes in pH levels of media following autoclaving. Although, the pH levels have not been measured following autoclaving, it is expected that these changes are likely to be relatively similar across the two pH treatments used.

It is noteworthy to indicate that auxin type used in the medium influenced culture morphology. Somatic embryos induced on 2,4-D were friable, translucent, yellowish-green in color, and globular to torpedo in shape. Somatic embryos induced on NAA were compact, opaque, pale-green in color, with an advanced morphology, forming cotyledon-like structures. Lazzeri et al. (1987) observed the same response and described somatic embryos initiated on NAA as having normal embryo morphology and development of adventitious roots. In the present study, adventitious roots were also observed on explants incubated on NAA, while no roots were observed on explants incubated on 2,4-D. Overall, explants incubated on 2,4-D produced higher amounts of friable callus than explants incubated on NAA. NAA produced most of the callus along the cut edges of the cotyledon, while 2,4-D generally produced callus over the entire surface of the cotyledon.

As somatic embryos initiated on NAA are more advanced in embryo morphology, they are not suitable for use in establishing repetitive suspension cultures. Liu et al. (1992) have reported that somatic embryos incubated in a medium containing NAA do not proliferate in suspension culture as well as those produced on a semi-solid medium containing 2,4-D.

Conclusions

In this study, a reliable system for establishing proliferative embryogenic cultures from three new genotypes of soybean was established by identifying optimal parameters for induction of somatic embryogenesis. Among the various parameters investigated, levels of sucrose and auxin in the culture medium were the most important, and they were significantly influenced by the soybean genotype used. This proliferative embryogenic culture system will be highly useful for developing effective transformation systems for these genotypes of soybean in order to improve such economic traits as disease and pest resistance, nematode resistance, or seed protein and/or oil content.

Acknowledgement

This project was funded by a grant received from the Illinois-Missouri Biotechnology Alliance.

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